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Comparison of three different methods for DNA extraction from bugs of family Pentatomidae (Hemiptera) for PCR

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Abstract
Developing a simple, time and cost efficient method for the extraction of DNA is essential when a large number of samples have to be processed. The success of PCR depends on the purity and quality of the DNA template and for this the method of specimen storage is equally critical as the DNA extraction methods. This study is an attempt to find the most suitable method of DNA extraction and the effect of killing method and storage conditions for extraction and purification of intact DNA in Pentatomid bugs.

Keywords: Intact DNA, Bugs, PCR

1. Introduction
The correct identification of species is very essential for evolutionary or phylogenetic analysis. Nowadays PCR based techniques are used for phylogenetic studies. For all these techniques, a key step is to obtain a good quality and quantity of genomic DNA from the specimens. But unfortunately not all specimens yield DNA of suitable quality because of its degradation during isolation. So, in molecular entomology, there is a constant need to develop a rapid, cost effective and efficient genomic DNA extraction protocol for large number of samples. Though several commercial genomic DNA extraction kits are available, they are generally either expensive or not readily available, especially for researchers in developing and under-developed countries around the world (1, 2). Standardized manual DNA extraction protocols are cheap and very efficient to collect DNA from samples. However, commercial kits are generally very fast but they generate large quantities of contaminant wastes, such as beads, filters, columns and micro centrifuge tubes. So, in this paper, different manual DNA extraction protocols have been tried for isolation of genomic DNA from pentatomid bugs with considerable attention on how specimens should be killed or preserved best and to find suitable method to successfully yield good quality and quantity of intact DNA. Family Pentatomidae, which has been chosen for the present work, is one of the largest families of the suborder Heteroptera, consisting of 4,722 species belonging to 896 genera (3). Majority of pentatomidae are plant feeders and several of them have great economic importance. Most of the economically important phytophagous bug species belong to this family and include majority of crop pests (4).

2. Materials and methods
Usually the insect samples contain a large amount of protein that hampers DNA extractions. The other problems usually encountered are small size of the insect and the presence of very thick exoskeleton. So, an ideal protocol is needed which should optimize DNA yield, minimize degradation and be efficient in terms of cost, time, labour and supplies (5). Variety of different protocols has been developed for extracting DNA both of higher quality and yield. However, the fundamentals of DNA extraction remain the same in all protocols. It is a multistep procedure which involves cell lysis by treatment with lytic enzymes and /or detergents, extraction with organic solvents and then the recovery of DNA by alcohol precipitations. Here, liquid nitrogen was used which allows access to DNA by breaking cell wall while other cellular enzymes and chemicals remain inactivated.

2.1 Phenol–Chloroform Isoamyl extraction method (6)
Homogenising buffer (Final pH = 8)

0.03M Tris HCL
0.01M EDTA (pH = 8.0)
0.1M NaCl
0.2M Sucrose
Lysing buffer (Final pH = 9.2)
0.25M EDTA (pH 8)
2.5% SDS
0.5M Tris HCl

1. Alcohol preserved specimens were rinsed in sterile distilled water briefly to remove excess alcohol.
2. Liquid nitrogen was used for crushing the samples in pestle mortar by adding 200-300 µL of Lysis buffer. Once sufficiently ground, 200 µL Homogenising buffer was added to it.
3. Finally 2 µL of Proteinase K was added. These samples were incubated at 55 °C for 4 hours to overnight.
4. Then 2µL RNase was added to each sample and kept at room temperature for 5-10 minutes. These were then centrifuged for 10 minutes at 12,000 rpm in a microcentrifuge.
5. The supernatant was carefully removed and poured into a fresh tube, avoiding pipetting of any pellet particles.
6. 500 µL of Phenol: Chloroform: Isoamyl alcohol (PCI) was added and centrifuged for 10 minutes. The top layer (aqueous phase) containing the DNA was then separated carefully from the lower organic layer containing proteins and fatty acids and was collected in a fresh Eppendorf tube.
7. 1:2 mixtures of chilled isopropanol and 100% ethanol were poured to this tube in volume equal to 2.5 times the volume of the supernatant. Then 5 µL of sodium acetate solution was added to this which further enhanced the DNA precipitation process.
8. These tubes were then placed at -20 °C for overnight or at -80 °C for 15 minutes. These were then centrifuged for 15 minutes at 12,000 rpm in a micro centrifuge.
9. Alcohol was carefully removed by pipetting or inverting the tube without disturbing the pellet and was dried on to the tissue paper upside down.
10. Then 70% ethanol was added and vortexed for a few seconds. Again these were centrifuged at 12,000 rpm in a micro centrifuge for 10 minutes.
11. The supernatant was again discarded and the DNA pellet was air dried and then dissolved in 50-70 µL TE buffer (depending on the amount of pellet obtained) and stored at -20 °C.

2.2 CTAB Based Extraction Method (7, 8)

CTAB is a cationic detergent which has the useful property of precipitating nucleic acids from homogenates at low ionic strength.

1. A mortar and pestle with the addition of liquid nitrogen was used for the grinding of fresh sample tissue into fine powder.
2. The ground tissue was transferred to a 1.5 mL eppendorf tube and it was homogenized in 1 mL of pre warmed (60 °C) CTAB extraction buffer (2% (w/v) CTAB diluted in 100 mM Tris-HCl, 20 mM EDTA, and 1.4 M NaCl; 0.2% (v/v) β-mercaptoethanol was added to the extraction buffer just prior to use.
3. Samples were incubated at 65 °C for 30 minutes with occasional mixing, and cooled to room temperature.
4. 2µL of RNase were added to the solution and incubated at 37 °C for 15 minutes.
5. One volume of Chloroform: isoamyl alcohol (24:1) was added, and the sample was emulsified by gentle inversion and centrifuged for 15 minutes at 13,000 rpm.
6. The top aqueous phase was transferred to a clean tube.
7. Two volumes of cold (-20 °C) 95% ethanol were added to the sample, mixed well and incubated at -20 °C until DNA strands were visible.
8. Again centrifuged at 13,000rpm for 15 minutes to collect the precipitate.
9. Discarded the supernatant and washed the pellet in 70% ethanol and then drained it dry.
10. Added TE buffer and stored it at -20 °C.

2.3 TNES Method (9)

Crushed 250 mg of tissue sample in autoclaved mortar pestle in presence of 1.0 mL of extraction buffer (Tris buffer 100 mM, EDTA 20 mM, NaCl 500 mM, Urea 7.0 M, 2% SDS and 0.1% β-Mercaptoethanol).

1. Centrifuged at 8000 rpm for 10 minutes and transferred the supernatant into the new eppendorf tube followed by addition of 1.0 mL each of phenol and chloroform in the ratio of 1:1.
2. Centrifuged at 8,000 rpm for 10 minutes. Transferred the upper aqueous layer in a new sterile eppendorf tube. Added 3.0M Sodium acetate (one tenth the volume of the sample) and ethanol (twice the volume of the sample) and mixed well.
3. Incubated the samples at -20 ºC overnight or 2 hours minimum for precipitation.
4. Centrifuged the samples at 10,000 rpm for 15 minutes, decanted the supernatant and washed the DNA pellet with 400 µL of chilled 70% ethanol.
5. Centrifuged it again at 10,000 rpm for 10 minutes and decanted the supernatant.
6. Air dried the DNA pellet aseptically for 15 minutes, dissolved in 50 µL of TE buffer and stored at -20 ºC for further experiments.

2.4 Spectrophotometric Analysis

Spectrophotometer was used to quantify the DNA and to check the purity. In general, the peak of UV absorption is at 260 nm for DNA and at 280 nm for protein. Thus, when a solution contains both DNA and protein, absorbance at 260 nm is mainly due to the DNA present, and absorbance at 280 nm is due to protein. A sample of DNA with the ratio of 1:8n is relatively free from protein contamination.

Table 1: Spectrophotometer analysis using three different protocols on two different species of bugs showing different DNA concentration

S. No.	Species	Extraction Methods	O.D. (260nm)	O.D. (280nm)	O.D. 260 /O.D. 280	DNA Conc.= O.D. 260 × 50 µg/ml × dilution factor
1.	*Halys dentatus*	Phenol chloroform isoamyl method	0.0732	0.0412	1.77	183
2.	*Halys dentatus*	CTAB method	0.0334	0.0299	1.17	83.5
3.	*Halys dentatus*	TNES method	0.0316	0.0290	1.08	79
4.	*Nezara antennata*	Phenol chloroform isoamyl method	0.0391	0.0355	1.10	97.7
5.	*Nezara antennata*	CTAB method	0.0341	0.0352	0.96	85.2
6.	*Nezara antennata*	TNES method	0.025	0.029	0.86	62.5

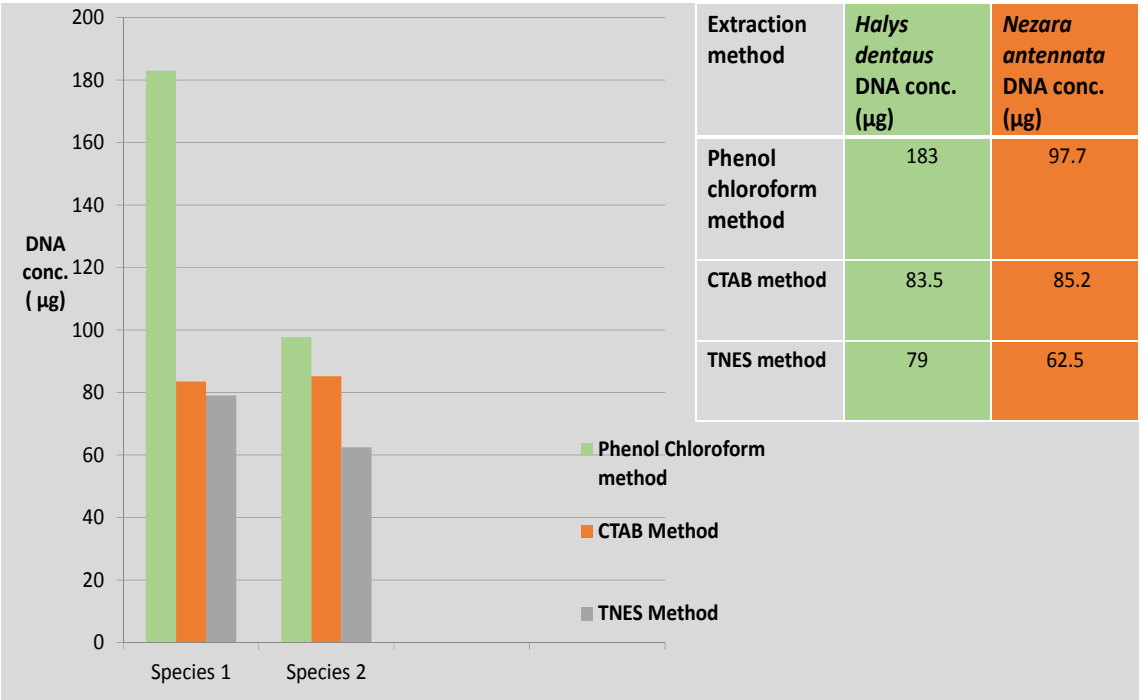

Fig 1: Graph indicating DNA concentration in two different species by using three different extraction methods

Table 2: Spectrophotometric analysis in three different species using PCI method

S. No.	Species	Method	DNA conc. (µg/ml)	260/280ratio
1.	*Halys dentatus*	PCI Method	70	1.11
2.	*Dolycoris indicus*	PCI Method	110	1.05
3.	*Nezara antennata*	PCI Method	160	1.10

Table 3: Spectrophotometric comparison in fresh and dried specimens

S. No.	Species	Method	Preservation	A1 (260nm)	A2 (280nm)	A1/A2	DNA conc.(µg)
1.	*Nezara antennata* (head)	PCI Method	Fresh	0.009	0.005	1.8	22.5
2.	*Nezara antennata* (head)	PCI Method	Air dried	0.007	0.005	1.4	17.5
3.	*Nezara antennata* (leg)	PCI Method	Fresh	0.010	0.007	1.42	25
4.	*Nezara antennata* (leg)	PCI Method	Air dried	0.005	0.032	1.6	12.5

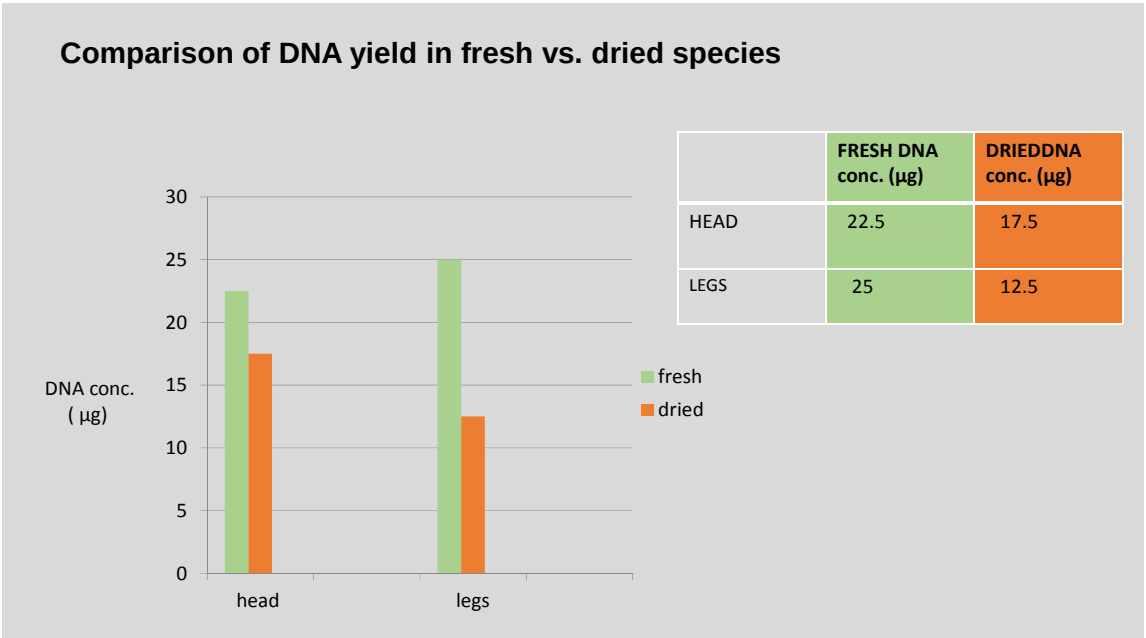

Fig 2: Graph indicating the DNA yield in fresh and air dried species

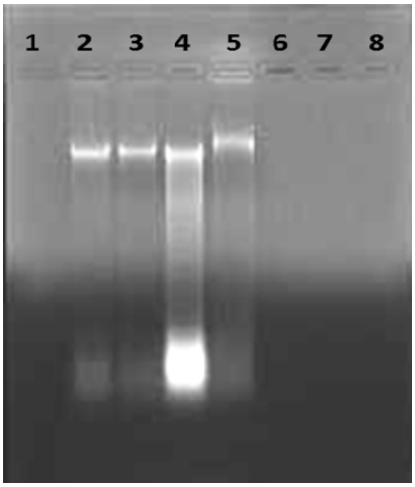

Fig 3: DNA isolated from four species by using Phenol Chloroform isoamyl method
Lane 2: *Halys dentatus*
Lane 3: *Dolycoris indicus*
Lane 4: *Nezara antennata*
Lane 5: *Nezara viridula*

2.5 DNA Amplification

The extracted DNA was amplified using universal primers, namely LCO1490 (5` GGTCAACAAATCATAAAGATATTGG3`) and HCO2198(5` TAAACTTCAGGGTGACCAAAAAATCA 3`)(10) which is known as Barcoding Region. All polymerase chain reactions were performed using Bio-rad T100TM thermal cycler. The thermal cycler conditions were the following: initial denaturation at 98 °C for 2 minutes followed by 40 cycles at 98 °C for 30 seconds, annealing at 50 °C for 40 seconds, elongation at 75 °C for 1 minute and final elongation at 75 °C for 7 minutes. 50 µl PCR cocktail constituted of Phusion DNA polymerase enzyme 1U/50 µl reaction, 5X Buffer 10 µl, 10 pm dNTP, 50 mM $MgCl_2$1 µl, 10 pm primers 1 µl each and MQ water (Thermo Fisher Scientific, India). 1-4 µl DNA was used for each PCR reaction, depending on the concentration of purified DNA.

2.6 Electrophoresis

PCR products were detected by gel electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

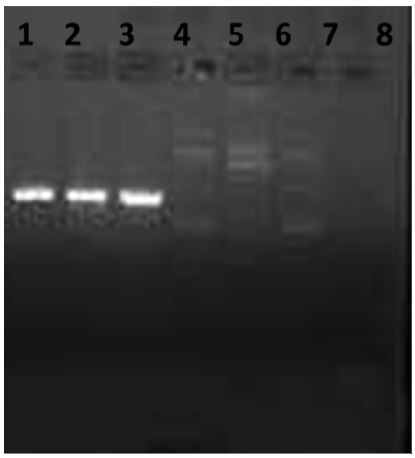

Fig 4: Gel showing DNA amplification bands in fresh samples (Lane 2-4) and no clear band in dried samples (Lane 5-7) by using Phenol chloroform isoamyl method.

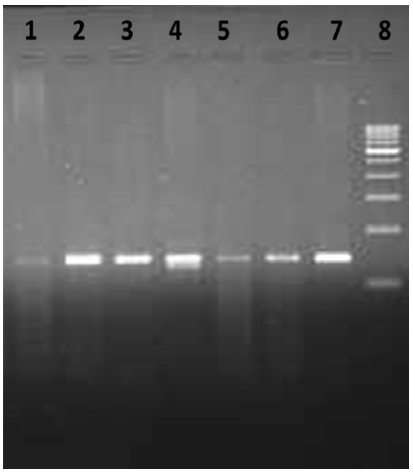

Fig 5: Amplified Product from fresh samples by Phenol chloroform isoamyl method.
Lane 1-7: Fresh samples
Lane 8: Ladder (500 base pair)

3. Results and Discussion

The aim of this study was to find a simple method of DNA extraction for Pentatomid bugs which could give us maximum yield and high quality of DNA suitable for further PCR applications. The method of DNA preservation also plays an important role in DNA extraction. In this study it was found that the specimens which were killed in ethyl acetate vapours and then air dried gave very less DNA yield as compared to samples which were preserved in 95% alcohol. Transparent to very light white pellet color in PCI method indicated very low protein contamination. However, light to dark brown pellet color in other methods indicated protein and other organic contamination. PCI method was found to be much more efficient in isolation of gDNA as compared to others.

Another point of focus was to know how different storage methods affect the quality and the quantity of DNA. It was found that DNA extracted from two-year-old specimens was more sheared than that from freshly assayed ones. DNA degradation in dried samples depends upon various factors like presence of free water, oxygen, heat and time of collection and their preservation method. The insect specimens who were killed in ethyl acetate or KCN gave very less yield as compared to specimens which are freshly collected and preserved in 95% alcohol. It has been observed that ethyl acetate has a tendency to vandalize DNA and so it should not be used for insect killing, otherwise it may hamper DNA yield (Dillon *et al*. 1996).

4. Conclusion

It can be concluded that all three protocols were suitable for DNA extraction but PCI method gave much more yield and high quality of DNA as compared to other methods. Moreover, the samples which were fresh and stored in 95% ethanol provided high DNA yield then the air dried samples. The proper method of sample storage is the most important step for DNA extraction.

When liquid nitrogen was used for DNA extraction, it enhanced the DNA yield. It is apparent that PCI method is a good one to obtain high quality DNA without the necessity of further steps of proteolysis and purification. It leads to isolation of good quantity of DNA without protein contamination which can be used for PCR amplification and further downstream applications.

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